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AUTHOR(S):

Hayashi, Masafumi; Kawaguchi, Takamasa;
Durcova-Hills, Gabriela; Imai, Hiroshi

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REVIEW ARTICLE

WILEY

Reproductive Medicine and Biology

Generation of germ cells from pluripotent stem cells in mammals

Masafumi Hayashi¹  | Takamasa Kawaguchi^{1,2} | Gabriela Durcova-Hills¹ | Hiroshi Imai¹ 

¹Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

²The Fukui Research Institute, Ono Pharmaceutical Company, Ltd., Fukui, Japan

Correspondence

Hiroshi Imai, Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

Email: imai@kais.kyoto-u.ac.jp

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Abstract

Background: The germ cell lineage transmits genetic and epigenetic information to the next generation. Primordial germ cells (PGCs), the early embryonic precursors of sperm or eggs, have been studied extensively. Recently, in vitro models of PGC induction have been established in the mouse. Many attempts are reported to enhance our understanding of PGC development in other mammals, including human.

Methods: Here, original and review articles that have been published on PubMed are reviewed in order to give an overview of the literature that is focused on PGC development, including the specification of in vivo and in vitro in mice, human, porcine, and bovine.

Results: Mammalian PGC development, in vivo and in vitro, have been studied primarily by using the mouse model as a template to study PGC specification in other mammals, including human, porcine, and bovine.

Conclusion: The growing body of published works reveals similarities, as well as differences, in PGC establishment in and between mouse and human.

KEYWORDS

embryonic stem cells, germ line, induced pluripotent stem cells, primordial germ cells, specification

1 | INTRODUCTION

The germ cell lineage transmits genetic and epigenetic information to the following generations. Primordial germ cells (PGCs), the early embryonic precursors of the germ cell lineage, differentiate into oocytes in females or sperm in males. To ensure that the appropriate epigenetic information is transmitted across generations, PGCs undergo extensive epigenetic modifications, including chromatin modifications and global erasure of DNA methylation. These complex changes are essential for the formation of healthy gametes. Understanding the mechanisms of the germ cell lineage development is crucial in reproductive medicine for the development of therapies and to treat

fertility problems in humans. It is also critical in understanding animal reproduction in order to sustain healthy and high-quality livestock. A thorough understanding of germ cell lineage development also might contribute to the preservation of endangered species for future generations.

In this review, the research that is studying PGC development in vivo in the mouse is discussed, as well as the successful attempts to derive PGCs from pluripotent stem cells, which also give rise to sperm or eggs in vitro. The recent progress made in studying PGCs in vivo in human is noted, as well as in establishing in vitro human models. Finally, reports about PGC development in domestic species, such as pig and bovine, are discussed.

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2 | PRIMORDIAL GERM CELL DEVELOPMENT IN VIVO IN THE MOUSE

The mouse is the best-studied research model for investigating the mammalian germ cell lineage. It has been established in gene knockout mice that the PGCs are induced from a competent epiblast by bone morphogenetic protein (BMP) and WNT3 signaling pathways.¹⁻⁴

Early embryonic development, leading to PGC induction, is described in Figure 1. After fertilization, the mouse zygotes undergo a series of cell divisions that lead to the formation of blastocysts at embryonic day 3.5 (E3.5). The blastocysts are composed of the pluripotent inner cell mass (ICM) and an outer monolayer of differentiated trophectoderm cells. Later, the ICM separates into a pluripotent epiblast and the differentiated primitive endoderm. At ~E4.5, the mouse blastocyst implants to the uterine wall. After implantation, at E5.5, the mouse embryo is rapidly developed and consists of extraembryonic ectoderm (ExE) that is derived from the trophectoderm, visceral endoderm (VE) that is derived from the primitive endoderm, and the epiblast. At this stage, WNT3 is first secreted by the posterior VE and is followed by WNT3 secretion by the posterior epiblast at E5.75. The WNT3 mutants have defects in gastrulation and the mutant epiblast does not form PGCs.^{1,5} At E6.0, BMP4 and BMP8b are secreted by ExE towards the proximal epiblast, inducing a few cells that become PGCs. In mice, BMP signaling is required for both mesoderm development and PGC specification.^{3,6,7} At the same time, dickkopf 1 and Cerberus 1 are secreted by the anterior VE, which prevents the anterior epiblast from PGC formation by inhibiting both WNT3 and BMP4 signaling.¹ At E7.25, ~40 PGCs are identified at the base of the incipient allantois.⁸ Then, the proliferating PGCs migrate towards the genital ridges through developing hindgut endoderm. During early migration, from E8.0 to E9.5, the PGCs undergo extensive epigenetic reprogramming, including the

remodeling of histone modification (for example, a decrease in histone H3 lysine 9 dimethylation [H3K9me2], an increase in histone H3 lysine 27 trimethylation [H3K27me3], and the reactivation of the inactive X chromosome in females that have been deactivated in the epiblast. At E11.0, the PGCs colonize the genital ridges, where major epigenetic reprogramming occurs, including genome-wide DNA demethylation of the repetitive sequences and differentially methylated regions of the imprinted genes.⁹ At this stage, the PGCs do not exhibit sex-specific imprints between the male and the female. Proliferating PGCs in the genital ridges reach ~25,000 cells. After E13.5, the PGCs enter meiosis in the female and are maintained in meiotic arrest during embryo development. Female-specific imprints are established after birth. In contrast, in the male, the PGCs enter mitotic arrest in G0/G1 and establish new male-specific imprints during gonocyte development.⁹

Both BMP4 and WNT3 signaling induce PGCs in the mouse. One of the WNT3 downstream targets is the T gene, known also as BRACHYURY, which promotes the expression of somatic (eg Hoxa1, Hoxb1, Snai1) and germ cell (BLIMP1 and PRDM14) genes.^{10,11} The BMP4 suppresses the somatic gene expression and prompts the up-regulation of the germ cell genes. The T induces the expression of BLIMP1 and PRDM14 in approximately six cells of the most proximal epiblast at E6.25.^{10,12} After the induction of these genes, BLIMP1 also promotes TFAP2C expression.^{13,14} The BLIMP1, PRDM14, and TFAP2C have a crucial role in PGC induction. The BLIMP1 inhibits DNA methylation in the induced genes and somatic cell genes together with TFAP2C. In contrast, PRDM14 promotes germ-cell specific and pluripotency-related genes with TFAP2C.¹⁵ The BLIMP1 acts on the inhibition of target genes and PRDM14 acts on the activation of target genes. It was revealed that NANOG induces BLIMP1 expression independently with the WNT3 or BMP4 signaling pathway.¹⁶ The activation of BLIMP1 by NANOG is inhibited by SOX2, which prohibits

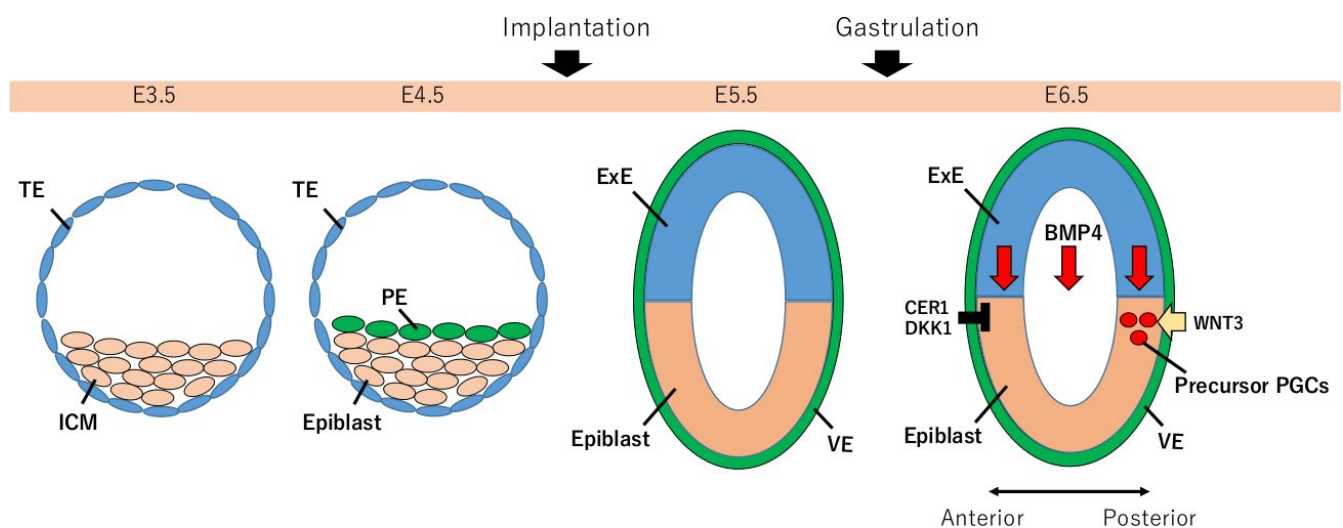


FIGURE 1 Specification of primordial germ cells (PGCs) in vivo in the mouse. At gastrulation, embryonic day 6.5 (E6.5), the mouse embryo forms an egg cylinder with anterior-posterior (A-P) axes. Both bone morphogenetic protein 4 (BMP4) from the extraembryonic ectoderm (ExE) and WNT3 from the posterior visceral endoderm (VE) induced few PGCs. Cerberus 1 (CER1), an antagonist of BMP4, and dickkopf1 (DKK1), an antagonist of WNT3, prevent the PGCs' induction at the anterior epiblast. ICM, inner cell mass; PE, primitive endoderm; TE, trophectoderm

embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) from differentiation into PGCs.

3 | INDUCTION OF PRIMORDIAL GERM CELL-LIKE CELLS IN VITRO IN THE MOUSE

There are two forms of pluripotency: the naïve form that is represented in vitro by ESCs that are derived from the inner cell mass of the blastocyst. The primed form is represented by epiblast stem cells (EpiSCs) that are derived from the primed E5.5-6.5 epiblast. The characteristics of naïve mouse ESCs are their unrestricted potential to contribute to chimeras, including the germ line, activity of both X chromosomes in the female, the expression of both naïve pluripotency genes (*Klf2*, *Klf4*, *Klf5*, *Esrrb*, *Tfcp2l1*, *Tbx3*, *Zfp42*), and core pluripotency genes (*Oct4*, *Sox2*, *NANOG*). The primed EpiSCs display many characteristics of the postgastrulation epiblast. They have one inactive X chromosome in the female, their ability to contribute in chimeras is limited, and both primed transcription factors (*Otx2*, *Oct6*, *Sox2*, *Tead2*, *Bex1*) are expressed together with core pluripotency factors (*Oct4*, *Sox2*, *NANOG*).^{17,18}

In initial attempts, the ESCs have been used for the derivation of PGCs through embryoid body formation;¹⁹⁻²² however, the efficiency of PGC induction was low, even when BMP4 was added to the cultures.²¹ Later, EpiSCs were used for the derivation of PGC-like cells (PGCLCs) in vitro. It was assumed that the EpiSCs are an in vitro equivalent of the epiblast, from which PGCs are specified. However, in the studied EpiSC colonies, <1.5% of the BLIMP1-positive cells also co-expressed Stella, a PGC-specific gene marker.²³ This experiment determined that EpiSCs are not sufficient to generate PGCLCs in vitro.

A two-step approach has been developed to induce PGCLCs from ESCs (Figure 2).²⁴ In the first step, the ESCs are cultured in the presence of basic fibroblast growth factor (bFGF) and activin A for 48 hours, thus converting them into EpiSC-like cells (EpiLCs). Both bFGF and activin A are used for the maintenance of EpiSCs in vitro. In the second step, between 800 and 2000 EpiLCs are cultured in the presence of leukemia inhibitory factor (LIF), stem cell factor (SCF), epidermal growth factor (EGF), and BMP4 for 4-6 days. In these conditions, the EpiLCs form aggregates within which a robust induction of PGCLCs has been observed. The efficiency of PGCLC induction was 30% higher than that observed in the EpiSCs.²³ Moreover, a transcriptome analysis confirmed that in vitro-generated PGCLCs are comparable to in vivo PGCs. This work suggests that PGCLC induction probably occurred during the transition from ESCs to EpiSCs.

In another method, PGCLCs were induced by the overexpression of master genes that are involved in the PGC specification. The overexpression of *Blimp1*, *Tfap2c*, and *Prdm14* in the EpiLCs generated the PGCLCs efficiently when only *Prdm14* was overexpressed.^{16,24} Moreover, it was shown that there was an overexpression of *NANOG* alone in the EpiLC-induced PGCLCs, even without the presence of BMP4.²⁵

4 | DIFFERENTIATION OF THE PRIMORDIAL GERM CELL-LIKE CELLS INTO SPERM AND EGGS IN VITRO

The PGCLCs exhibit transcriptome (expression of pluripotency and germ-cell specific genes) and epigenetic properties (chromatin and DNA modifications) that are associated with the migrating PGCs in the mouse embryo. Therefore, the ability of mouse PGCLCs to differentiate further into sperm or oocytes has been studied in both males and females by many researchers (Figure 2).

For the in vitro reconstruction of the male pathway, several approaches have been applied. In one approach, only the BLIMP1- and STELLA-positive PGCLCs were transplanted into the neonatal testes that lacked endogenous germ cells. The transplanted PGCLCs underwent normal spermatogenesis and formed sperm,²⁴ which when injected into wild-type oocytes developed into blastocysts. The transplanted blastocysts gave rise to viable offspring that had a normal sex-specific methylation status in the analyzed imprinted genes. In contrast, the unsorted PGCLCs for BLIMP1 and STELLA expression gave rise to only tumors after their injection into neonatal testes.

In the second approach, the PGCLCs were differentiated into spermatogonia-like cells.²⁶ Day 4 PGCLCs were mixed together with gonadal somatic cells, isolated from E12.5 embryonic gonads, to form aggregates. Then, the aggregates were cultured under floating conditions for 2 days, followed by placing them on permeable membranes for a gas-liquid culture. After 21 days, the cells that expressed either GATA4 and SOX9 (Sertoli cells marker) or DDX4 (gonadal germ cell marker) and PLZF (spermatogonial stem cell marker in pro-spermatogonia) were identified in the seminiferous tubule-like structures. The DDX4- and PLZF-positive cells gave rise to cell lines that resembled germline stem cells when cultured under appropriate conditions. When injected into the adult testis, the cells differentiated into fertile sperm.

In another report, PGCLCs that were co-cultured with neonatal testicular somatic cells in the presence of appropriate factors differentiated into spermatid-like cells.²⁷ The Blimp1- and Stella-positive PGCLCs were mixed with postnatal testicular somatic cells and cultured for 6 days in the presence of retinoic acid (RA), BMP2, BMP4, BMP7, and activin A. Once Stra8-expressing cells, a meiosis marker, were detected, the cultures were exposed to a combination of three sex hormones (bovine pituitary extract, testosterone, and follicle-stimulating hormone [FSH]) in order to progress the Stra8-positive cells into haploid spermatid-like cells. First, the haploid cells were identified at ~day 10, with numbers increasing over the next 4 days. The achieved efficiency rate was between 14% and 20%. When the in vitro spermatid-like cells were injected into wild-type oocytes, fertile offspring were born.

For the reconstruction of oogenesis in vitro, several approaches have been reported. In one method, the reconstructed ovaries were made of female PGCLCs that were mixed with female gonadal somatic cells that had been depleted of PGCs and were implanted into the recipient ovaries.²⁸ After 4 weeks, the secondary oocytes were isolated from the reconstructed ovaries and fertilized with wild-type sperm to

Mouse

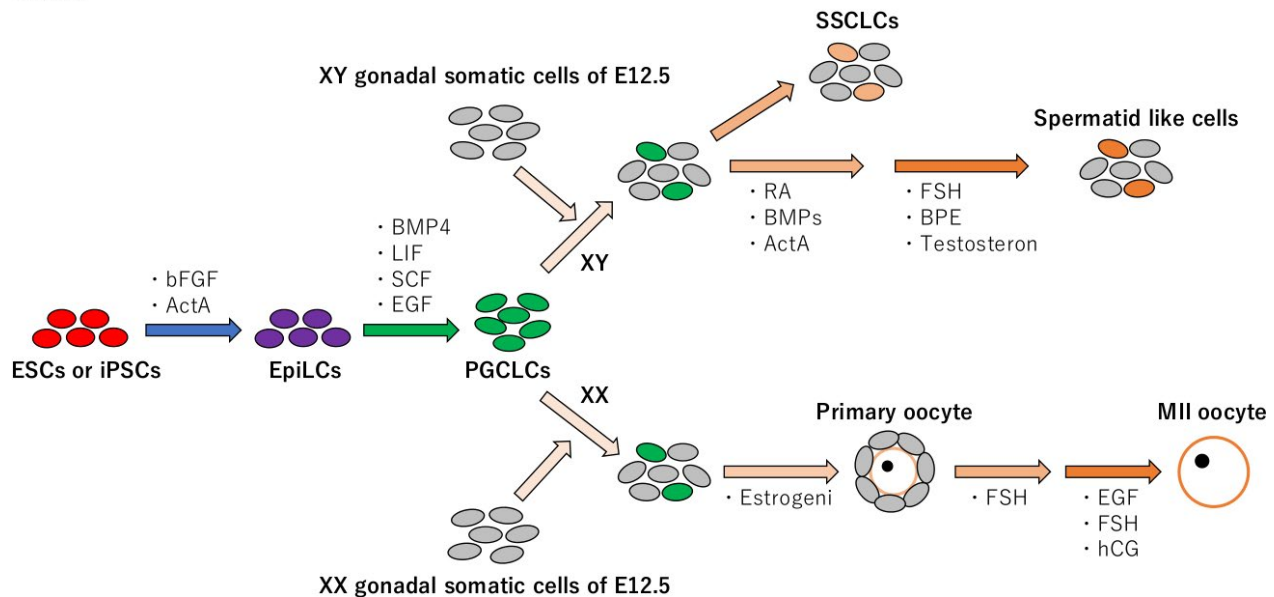


FIGURE 2 In vitro induction of germ cells in the mouse. Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) that had been cultured in the presence of basic fibroblast growth factor (bFGF) and Actin A are converted into epiblast-like cells (EpiLCs). Primordial germ cell-like cells (PGCLCs) are induced from EpiLCs by exposing them to bone morphogenetic protein 4 (BMP4), leukemia inhibitory factor (LIF), stem cell factor (SCF), and epidermal growth factor (EGF). Male (XY) PGCLCs that are mixed together with male gonadal somatic cells that have been isolated from E12.5 genital ridges form aggregates that differentiate into spermatogonial stem cell-like cells (SSCLCs). When the aggregates are exposed first to retinoic acid (RA), BMPs, and Actin A, followed by follicle-stimulating hormone (FSH), bovine pituitary extract (BPE), and testosterone, the PGCLCs differentiate into spermatid-like cells. Female (XX) PGCLCs are mixed together with female gonadal somatic cells that have been isolated from E12.5 genital ridges. The aggregates, which are cultured in the presence of an estrogen inhibitor, differentiate into primary oocytes, which in the presence of FSH and later in the presence of EGF, FSH, and human chorionic gonadotropin (hCG), give rise to MII oocytes

produce viable offspring. However, the efficiency of this approach was very low (3.9%) due to the failure of the PGCLC-derived oocytes and zygotes to extrude the second polar body.²⁸

Recently, an improvement of the previous approach has been reported.^{11,29} The reconstructed ovaries were cultured in the presence of an estrogen inhibitor in order to prevent multi-oocyte formations in one follicle.¹³ After 21 days of culture, granulosa cells were detected around the primary oocytes that had developed from the PGCLCs. This was followed by mechanical separation of the secondary follicle-like structures in the presence of FSH for 11 days, when complexes of the primary oocyte and granulosa cells were observed. The observed granulosa cells proliferated and cumulus-oocyte complexes (COCs) were formed. After in vitro maturation of the COC, the first polar body was extruded and caused ovulation as secondary oocytes. When these oocytes were fertilized with wild-type sperm, they produced zygotes that developed into healthy offspring. The epigenetic status of the induced oocytes from the PGCLCs showed normal methylation in the examined imprinting genes. However, the induced oocytes, in contrast to the control, exhibited a low rate of fertilization and chromosomal abnormalities.

These recent advances in the development of an in vitro model of oogenesis are encouraging; however, there are several obstacles to investigate. One of them is a need for the co-culture of PGCLCs with embryonic gonadal somatic cells to differentiate the PGCLCs into oocytes or sperm.

5 | INDUCTION OF HUMAN PRIMORDIAL GERM CELL-LIKE CELLS IN VITRO

The progress that has been made by studying mouse PGC development, both in vivo and in vitro, has led researchers to attempt to induce human PGC-like cells (hPGCLCs) from PSCs. Studies on hPGCs are difficult due to the inaccessibility of human embryos for research studies. Therefore, the accurate timing of hPGC specification cannot be determined precisely, but it is assumed to occur at around gastrulation in the second or third week of development.³⁰

In human, specified PGCs first were identified by displaying their characteristic morphology by using transmission electron microscopy. The hPGCs were observed in the endoderm of the yolk sac wall near the allantois during the fourth week, or embryonic day 24 (E24). By the sixth week (E37), they migrate through the embryo into the genital ridges, embryonic precursors of the testes or ovaries. During the migration and colonization of the genital ridges, the hPGCs proliferate until the tenth week, when they enter either mitotic quiescence in the XY embryos or meiotic prophase in the XX embryos.^{30,31} Several studies have revealed that the hPGCs express AP,³² OCT4/POU5F1,³³⁻³⁶ NANOG,^{35,36} BLIMP1/PRMT5,³⁷ c-KIT,³⁴ DDX4/VASA,^{38,39} DAZL,^{35,39} and LIN28.⁴⁰ However, the studies also showed that the hPGCs, in contrast to mPGCs, do not express SOX2, the core pluripotency gene in the mouse.^{36,41} Recent RNA sequencing studies that have been performed on hPGCs⁴²⁻⁴⁴ highlight the similarities between

mouse and human PGCs at comparable stages of PGC development, but also demonstrated unique features of hPGCs from mPGCs. The hPGCs express SOX17, which is an endoderm regulator, and TEAD4, a trophoblast regulator. The crucial role of SOX17 in hPGC specification is established.⁴⁵

Recent efforts in generating hPGCs in vitro have been reported (Figure 3). The protocol of mPGCLC induction from ESCs or iPSCs²⁴ was used as a template for inducing hPGCLCs. However, the achieved efficiency of hPGCLC induction from hiPSCs is <5% and from EpiLCs ~30%.⁴⁵⁻⁴⁷ It was assumed that the low level of efficiency was a result of the prime state of human ESCs or iPSCs. Therefore, a new protocol was developed, in which hESCs were converted into a naïve form by exposing them to “4i” inhibitors. First, the hESCs are converted into a naïve state by growing them in the presence of the inhibitors of four kinases (glycogen synthase kinase [GSK]-3 β , extracellular signal-regulated kinase [ERK]1/2, p38, and c-Jun N-terminal kinase) and in culture media that is supplemented with bFGF, LIF, and transforming growth factor (TGF)- β .⁴⁸ Then, the naïve hESCs are cultured in low-adhesion dishes in the presence of BMP4, LIF, SCF, and EGF for ~8 days. At ~30% of NANOS3- and TNAP-tissue non-specific AP, positive hPGCLCs are induced within 4-5 days of culture. The global gene expression profile of the hPGCLCs matched with that profile of 7 weeks' old hPGCs in vivo.⁴⁵

In an alternative approach, human ESCs or iPSCs first are converted into incipient mesoderm-like cells (iMeLCs) by an exposure to activin A and GSK-3 β inhibitor.⁴⁹ This is followed by culturing iMeLCs in the presence of BMP4, LIF, SCF, and EGF in a low-adhesion culture for 4 days. The formed aggregates comprise ~30% of the BLIMP1- and TFAP2C-positive hPGCLCs. The in vitro-generated hPGCLCs, using the two protocols described above, are transcriptionally similar⁴⁹ and they do not express DDX4 and DAZL, markers of late PGCs in vivo.

It is important to highlight that the comparison between mouse and human PGCLCs showed that PGC specification in the mouse and human differ. In the mouse, BLIMP1 is the master gene to induce PGCs. In contrast, in human, BLIMP1 expression is controlled

by SOX17.⁴⁵ SOX2, an essential gene for mPGC development, is not detected in human PGCs.^{36,50} Several challenges lie ahead of evaluating the developmental potential of hPGCLCs. First is to determine the ability of hPGCLCs to further differentiate into late PGCs. If this proves to be successful, then the second challenge is to differentiate them into mature sperm or oocytes in vitro. Some studies have shown that male germ cell lineages, including postmeiotic, spermatid-like cells, are induced from hESCs and hiPSCs.^{47,51,52} However, complete spermatogenesis in vitro has not been accomplished yet in human.

6 | INDUCTION OF PRIMORDIAL GERM CELL-LIKE CELLS IN NON-MURINE MAMMALS

The early stages of porcine PGC (pPGC) development appears to be similar to that of the mouse. The PGCs are identified in E12-E14 embryos by OCT4, BLIMP1, and SOX17 expression, pluripotency markers of PGCs.⁵³⁻⁵⁵ Then, the pPGCs at E15, stage-equivalent to the E8.5 in the mouse, commence migration towards the genital ridges and, at E18, they start to colonize the genital ridges.⁵⁶ Recently, the induction of porcine PGCLCs (pPGCLCs) from porcine iPSCs was reported (Figure 4).⁵⁷ Porcine iPSCs first are converted into EpiLCs in the presence of bFGF and activin A for 2 days. Then, the disaggregated cultures are kept in the presence of four cytokines (BMP4, LIF, SCF, and EGF) to induce pPGCLCs. The robust induction of pPGCLCs has been detected after only 4 days of culture, confirmed by the expression of BLIMP1, PRDM14, and STELLA, the germ-cell specific genes. However, DDX4, a late PGC marker, is only moderately upregulated. Several imprinted genes in the pPGCLCs become demethylated, suggesting that they initiate the imprint erasure. Histone modification dynamics at H3K9me2 and H3K27me3 in the pPGCLCs agree with the histone modifications that are observed in the pPGCs that have been isolated from E15 old embryos. Spermatogonial stem cell-like

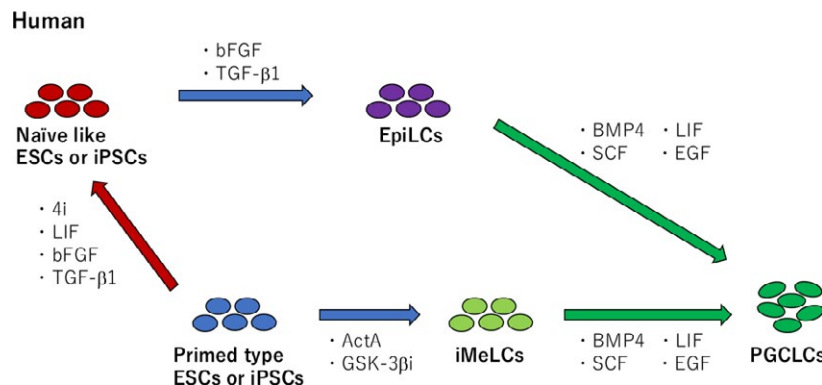


FIGURE 3 In vitro induction of primordial germ cell-like cells (PGCLCs) in human. Primed-type embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) are cultured in the presence of Actin A and glycogen synthase kinase (GSK)-3 β i, giving rise to induced mesoderm-like cells (iMeLCs). The cultured iMeLCs, in the presence of bone morphogenetic protein (BMP)4, leukaemia inhibitory factor (LIF), stem cell factor (SCF), and epidermal growth factor (EGF), give rise to PGCLCs. In another approach, primed-type ESCs or iPSCs are converted into naïve-like ESCs or iPSCs by culturing them in the presence of BMP4i, LIF, basic fibroblast growth factor (bFGF), and transforming growth factor (TGF)- β 1. Then, the naïve ESCs or iPSCs are cultured in the presence of bFGF and TGF- β 1, giving rise to EpiLCs, which are induced into PGCLCs by culturing them with BMP4, LIF, SCF, and EGF. 4i, four inhibitors

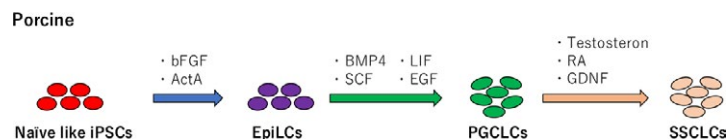


FIGURE 4 In vitro induction of germ cells in the pig. Naïve-like induced pluripotent stem cells (iPSCs) are cultured in the presence of basic fibroblast growth factor (bFGF) and Actin A, giving rise to epiblast-like cells (EpiLCs), which are exposed to bone morphogenetic protein (BMP)4, leukaemia inhibitory factor (LIF), stem cell factor (SCF), and epidermal growth factor (EGF) and differentiate into primordial germ cell-like cells (PGCLCs). These then differentiate further into spermatogonial stem cell-like cells (SSCLCs) by culturing them with testosterone, retinoic acid (RA), and glial cell line-derived neurotrophic factor (GDNF)

cells are induced from the pPGCLCs by the presence of RA, glial cell line-derived neurotrophic factor, and testosterone. Both DAZL and STRA8 have been detected.

Bovine PGCs are identified by AP staining in E18–E39 old embryos.^{58,59} The AP-positive PGCs are observed in the proximal yolk sac, in the hindgut and midgut, and in the genital ridges.^{58,59} Recently, bovine iPSC lines have been derived.^{60–65} When the bovine iPSCs are converted into a naïve state of pluripotency, they contribute many tissues in the chimera, including gonads.⁶² Bovine-primed iPSCs have been induced into bovine PGCLCs (bPGCLCs) via embryoid body formation and culture in the presence of RA and BMP4.⁶⁶ However, this approach to induce bPGCLCs is ineffective and is based on the detection of DDX4.

7 | CONCLUSION

The mouse is the best-studied model of PGC development. In vitro, it has been demonstrated that naïve PSCs, ESCs, or iPSCs are the most efficient source of PGCLC induction. Moreover, when PGCLCs are cultured under appropriate in vitro conditions, they have the capacity to differentiate further to form either oocytes or sperm. In contrast to the mouse, only the primed-type iPSCs are available in other mammals. It is going to take time and effort to determine the conditions under which conversion from the primed type into the naïve pluripotent stem cells can be established in non-murine mammals. A recent successful report on effective hPGCLC induction from human iPSCs might be used as a template to develop strategies for other species. The work also suggests that modified culture conditions must be developed for every species. This in vitro approach also might help to understand the differences in the development and differentiation of germ cell lineage among a variety of species, including domestic animals and endangered species.

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DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. **Human and Animal Rights:** The protocol for the research project, including human participants, was approved by a suitably constituted ethics

committee. This article does not contain any study with animal participants that was performed by any of the authors.

ORCID

Masafumi Hayashi <http://orcid.org/0000-0003-3286-9029>

Hiroshi Imai <http://orcid.org/0000-0003-3702-2708>

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